Insulin-Like Growth Factor-1 Controls Type 2 T Cell-Independent B Cell Response

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The IGF-1 receptor (IGF-1R) is expressed on T and B lymphocytes, and the expression of the insulin- and IGF-1-signaling machinery undergoes defined changes throughout lineage differentiation, offering a putative role for IGF-1 in the regulation of immune responses. To study the role of the IGF-1R in lymphocyte differentiation and function in vivo, we have reconstituted immunodeficient RAG2-deficient mice with IGF-1R$^{-/-}$ fetal liver cells. Despite the absence of IGF-1Rs, the development and ex vivo activation of B and T lymphocytes were unaltered in these chimeric mice. By contrast, the humoral immune response to the T cell-independent type 2 Ag 4-hydroxy-3-nitrophenyl acetyl-Ficoll was significantly reduced in mice reconstituted with IGF-1R-deficient fetal liver cells, whereas responses to the T cell-dependent Ag 4-hydroxy-3-nitrophenyl acetyl-chicken globulin were normal. Moreover, in an in vitro model of T cell-independent type 2 responses, IGF-1 promoted Ig production potently upon polyvalent membrane-IgD cross-linking. These data indicate that functional IGF-1R signaling is required for T cell-independent B cell responses in vivo, defining a novel regulatory mechanism for the immune response against bacterial polysaccharides. ***The Journal of Immunology*****, 2005, 174: 5516–5525.**

The insulin-like growth factor receptor (IGF-1R)[2] is a receptor protein tyrosine kinase, sharing structural and functional homologies with the insulin receptor (IR) (1). Ligand binding to either IR or IGF-1R leads to autophosphorylation of tyrosine residues in the cytoplasmic regions of the receptor β subunits and subsequent recruitment and phosphorylation of insulin receptor substrate (IRS) proteins (2, 3). This finally results in activation of the Ras/Raf-MAPK pathway, on the one hand, and the PI3K/Akt/p70^{S6K} pathway, in contrast, among other effects leading to cell proliferation, differentiation, and rescue of cells from apoptosis (4–6).

IR and IGF-1R are expressed on many cell types, including B and T cells (7–9), and several studies have indicated a role for insulin and IGF-1 effects in lymphocyte development and function (10–19). IGF-1 receptors are present on T lymphocytes, and their expression is down-regulated through the course of differentiation; immature CD4/CD8 double-negative thymocytes show a 3- to 4-fold higher expression of IGF-1Rs than double-positive or single-positive cells (10). Additionally, IGF-1 was able to stimulate DNA synthesis in thymocytes in vitro, and it also led to an increase in transcription and protein synthesis of CD25 and IL-2, both markers for T cell activation (14). In fetal thymic organ cultures (FTOC), anti-IGF-1R Abs blocked the differentiation of T lymphocytes from pluripotent precursors (15). In contrast, IGF-1 exhibited synergistic effects with IL-4 in the induction of DNA synthesis in a pro-B cell line, indicating a role in B cell proliferation (19).

Moreover, several studies have shown the participation of IR and IGF-1R downstream signaling molecules in the proliferation and function of lymphocytes. IL-4, which is an important factor for B cell proliferation and survival, has been shown to induce phosphorylation of IRS-2 in resting B lymphocytes and subsequent recruitment of the regulatory subunit of the PI3K p85. Nevertheless, transgenic overexpression of IRS-2 in murine B cells had no effect on cell proliferation and protection from apoptosis in vivo (20).

Taken together, these findings suggest a role for IGF-1 signaling in the proliferation, differentiation, and function of lymphocytes. Studies of the role of IGF-1-related signaling in lymphocyte development and function, however, have been largely limited by the fact that they were performed in cultured cells in vitro.

To further characterize the role of IGF-1R signaling in vivo, it would be favorable to analyze mice lacking the receptor. IGF-1R-deficient mice die shortly after birth; hence, it is impossible to analyze lymphocyte differentiation and function in these mice (21). Therefore, we chose to reconstitute MLR2 (RAG2)-deficient C57BL/6 mice with fetal liver cells (FLC) derived from IGF-1R$^{-/-}$ and control embryos (22). Analysis of these chimeric mice showed that animals reconstituted with IGF-1R-deficient fetal liver cells develop lymphocyte subpopulations similar to wild-type (wt) reconstituted control animals. FTOC (23) of thymi derived from IGF-1R$^{-/-}$ embryos also revealed normal thymocyte development in the absence of functional IGF-1R. Surprisingly, immunization experiments revealed a decreased T cell-independent B cell response in IGF-1R$^{-/-}$ chimeras, whereas T cell-dependent responses were normal. This finding was consistent with the observation that IGF-1 can stimulate Ig production in an in vitro model for T cell-independent type 2 (TI-2) responses.

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Received for publication July 27, 2004. Accepted for publication February 14, 2005.

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[2] Abbreviations used in this paper: IGF-1R, insulin-like growth factor receptor; CG, chicken globulin; ED, embryonic day; FLC, fetal liver cell; FTOC, fetal thymic organ culture; Gab-1, Grb2-associated binder 1; IR, insulin receptor; IRS, insulin receptor substrate; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MZ, marginal zone; NP, 4-hydroxy-3-nitrophenyl acetyl; TI-2, T cell-independent type 2; wt, wild type.

0022-1767/05/$02.00

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUL 2004		**00-00-2004 to 00-00-2004**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Insulin-Like Growth Factor-1 Controls Type 2 T Cell-Independent B Cell Response	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University of the Health Sciences,Department of Pathology,Bethesda,MD,20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**10**	

These data show that lymphocyte IGF-1R expression is not required for lymphocyte development and differentiation, but is specifically required for T cell-independent B cell responses. Because such T cell-independent responses represent important defense mechanisms against bacterial Ags, our findings assign IGF-1R-mediated signaling a novel role in these immune defense processes.

Materials and Methods

Mice

IGF-1R$^{+/-}$ 129/Sv mice (21) were generously provided by A. Efstradiatis (Columbia University, New York, NY). RAG2-deficient mice (24) were purchased from Taconic Farms. Mice were kept in specific pathogen-free animal facilities according to institutional guidelines. DNA for typing was prepared from tail biopsies.

Cell sorting and semiquantitative RT-PCR

For sorting of lymphocyte subpopulations, single cell suspensions of spleen, thymus, and bone marrow of wt 129/Sv mice were prepared. According to their surface Ags, bone marrow-derived B lymphocytes were sorted into pro- and pre-B cells and immature and recirculating B cells (anti-B220/CD45R and anti-IgM) (25) splenic B lymphocytes were sorted into marginal zone (MZ) and follicular B cells (anti-B220/CD45R, anti-CD21, and anti-CD23) (26), and thymocytes were sorted into double-negative, double-positive, and CD4 and CD8 single-positive T cells (anti-CD4 and anti-CD8) (27). RNA isolation was performed using the RNeasy system (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Qiagen) and reverse transcribed with SuperScript II (Invitrogen Life Technologies). Twenty-five to 40 cycles of 30 s at 95°C, 45 s at 57°C (IGF-1R and IRS-2) or 60°C (GAPDH, IRS-1, and IR), and 45 s at 72°C were used for amplification using the following primers: IGF-1R 5′, 5′-CGA CGT ATG AGA ACT TCA TGC-3′; IGF-1R 3′, 5′-CAC ATG GTG ACA ATT GAA CTC C-3′; IR 5′, 5′-CAG CGA GCT GTT TGA GCT GG-3′; IR 3′, 5′-GGC CAG AGA TGA CAA GTG ACT-3′; IRS-1 5′, 5′-TGC GCA GGC ACC ATC TCA AC-3′; IRS-1 3′, 5′-GGA CAC GGA AGC ACT AGA GC-3′; IRS-2 5′, 5′-CTT GGA AGA GGA GAG ACT GGA-3′; IRS-2 3′, 5′-ATC CAT GGA GCC TAC TGT GTC-3′; GAPDH 5′, 5′-ACC ACA GTC CAT GCC ATC AC-3′; and GAPDH 3′, 5′-TCC ACC ACC CTG TTG CTG TA-3′. PCR products were resolved on 1% agarose gels in Tris-acetic acid-EDTA buffer, and the intensity of the bands was quantified using the QuantityOne gel documentation system (Bio-Rad).

To control for the purity of the bone marrow B cell subpopulations, expression of the following genes was also examined by PCR (28): TdT 5′, 5′-GAT TTC GAG ACT TGG TCC TCT TCA TTT TGG-3′; TdT 3′, 5′-CAA GGA ATC CCC TCT GTG TCT TTC ATG CTG-3′; Lambda5 5′, 5′-GAG ATC TAC ACT GCA AGT GAG GCT AGA G-3′; Lambda5 3′, 5′-CTT GGG CTG ACC TAG GAT TG-3′; sterile κ L chain 5′, 5′-CAG TGA GGA GGG TTT TTG TAC AGC CAG ACA G-3′; and sterile κ L chain 3′, 5′-CTC ATT CCT GTT GAA GCT CTT GAC AAT GGG-3′ in 35 cycles of 20 sec at 94°C, 15 s at 55°C, and 60 s at 72°C.

FLC transfers

Embryos were obtained by intercrossing IGF-1R$^{+/-}$ mice. IGF-1R$^{-/-}$ and wt FLC were isolated from embryos on day 16.5 postconception. Mice and embryos were genotyped by PCR analysis using the primers IGF-1R3 (5′-ATC ATC CTT ACC ACC CTC T-3′) and IGF-1R4 (5′-GGC ACC CTC AAA GTT TAG-3′). Thirty PCR cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C yielded a 536-bp wt band and a 1336-bp knockout band, respectively. For FLC transfer, single cell suspensions of fetal livers were prepared, and 10^6 FLC in HBSS in a volume of 300 μl were injected i.v. into 8-wk-old RAG2$^{-/-}$ C57BL/6 mice (24) that had been sublethally irradiated (500 rad) the previous day. Recipients were treated with antibiotics in the drinking water after irradiation; the mix of antibiotics was changed every 2 wk (kanamycin sulfate, neomycin sulfate, and bacitracin, or penicillin, streptomycin sulfate, and gentamicin sulfate; 30 mg/L each).

Cell staining and flow cytometry

Reconstitution was examined 4 and 5 wk after FLC transfer by staining PBL with anti-B220/CD45R (29), anti-IgM (30), anti-CD4 (31), anti-CD8 (32), anti-CD90 (33), and anti-CD5 (32) Abs. Six weeks after reconstitution, staining of surface markers and FACS analysis were performed on cells derived from spleen, peritoneal cavity, bone marrow, and lymph nodes. Single cell suspensions were prepared and incubated for 15 min at 10^6 cells/30 μl in staining buffer (PBS containing 0.5% BSA and 0.01% NaN_3) with optimal amounts of FITC-, PE-, biotin-, CyChrome-, or allophycocyanin-conjugated Abs. Abs were purchased from BD Pharmingen and Serotec; flow cytometric analysis was performed on a FACSCalibur cytometer (BD Biosciences).

Fetal thymic organ culture

IGF-1R$^{+/-}$ mice were mated, and the occurrence of vaginal plugs was defined as embryonic day (ED) 0.5. Embryos were prepared on ED 14.5–17.5, and fetal thymi were cultured up to 1 wk on Millicell culture plate inserts (Millipore) in DMEM-10 (DMEM; Life Sciences; 10% FCS, 6 ml of sodium pyruvate, 6 ml of nonessential amino acids, 6 ml of glutamine, and 50 μM 2-ME) at 37°C in 7.5% CO_2 and 100% relative humidity. For FACS analysis, thymic lobes were passed through a 30-μm pore size nylon mesh. Potentially migrated thymocytes were collected by centrifugation of the culture medium from the respective wells and rinsing the culture plate inserts with PBS. Cells were stained with anti-CD4 and anti-CD8 Abs as described above.

Analysis of protein expression and tyrosine phosphorylation

For the analysis of tyrosine phosphorylation, MACS-purified (34) B cells were suspended in RPMI 1640 supplemented with 1% FCS and stimulated with 20 μg/ml F(ab′)$_2$ of goat anti-mouse IgM or whole goat anti-mouse IgM for the indicated time at 37°C (35). Cells were pelleted and lysed in lysis buffer (50 mM HEPES, 1% Triton X-100, 50 mM NaCl, 0.1 M sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 10 μg/ml aprotinin, 2 mM benzamidine, 0,1% SDS, and 2 mM PMSF, pH 7.4), and the lysate was clarified by centrifugation for 15 min at 12,000 × *g*. Fifty micrograms of protein were resolved by SDS-PAGE on 8% polyacrylamide gels and blotted on Hybond ECL nitrocellulose membranes (Amersham Biosciences). After blocking with 1× blocking reagent (Roche), membranes were incubated with mouse anti-phosphotyrosine (PY99; Santa Cruz Biotechnology), Akt, or pAkt (Cell Signaling Technology and New England Biolabs) mAb and peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma-Aldrich). Bound Ab was then detected by ECL (Amersham Biosciences). For analysis of IGF-1R and IR protein expression, whole embryos were homogenized in lysis buffer, and lysates were processed as described above. After protein transfer, nitrocellulose membranes were incubated with anti-mouse IGF-1Rβ (C-20) or IRβ Ab (C-19; Santa Cruz Biotechnology) and HRP-conjugated anti-rabbit IgG (Sigma-Aldrich).

Analysis of B cell proliferation and up-regulation of activation markers

Splenic cells derived from chimeras were cultivated at 1.5×10^5 cells/well in 96-well plates and were stimulated with F(ab′)$_2$ of goat anti-mouse IgM or whole anti-mouse IgM (2.5 μg/ml; Dianova), hamster anti-mouse CD40 Ab (2 μg/ml) (BD Pharmingen), mouse rIL-4 (25 U/ml; R&D Systems), LPS from *Escherichia coli* serotype 055:B5 (20 μg/ml; Sigma-Aldrich), and human rIGF-1 (10^{-8} M; Sigma-Aldrich) in RPMI 1640 medium. After 48 h, cells were harvested and stained with PE- or FITC-conjugated Abs to B220/CD45R (RA3-6B2) to gate on B cells, CD86 (B7.2; BD Pharmingen) and MHCII (M5/114; BD Pharmingen) to assess the activation status (35).

For determination of proliferation, splenic B cells were purified by depletion of non-B cells with anti-CD43 Ab coupled to magnetic beads and MACS columns (Miltenyi Biotec) as described previously (34), plated on 96-well plates at 10^5 cells/well, and stimulated with 0.1, 1, or 10 μg/ml F(ab′)$_2$ of goat anti-mouse IgM, hamster anti-mouse CD40 (36), or LPS and 0.1, 1, or 10 ng/ml dextran-coupled anti-IgD alone or in combination with IL-4 (25 U/ml), IL-5 (0.1 ng/ml), or IGF-1 (10^{-7} M) for 72 h in RPMI 1640 medium supplemented with 10% FCS (35, 37). After addition of 0.5 μCi [*methyl*-^{3}H]thymidine/well (Amersham Biosciences), cells were incubated for 12–16 h, harvested with a cell harvester (Packard Instruments), and bound to a filter. Incorporated ^{3}H was measured in a beta counter (Beckman Coulter). All experiments were performed in triplicate.

Immunization of reconstituted mice

The ability of chimeric mice to mount a humoral immune response was determined by immunization of IGF-1R$^{-/-}$ and wt reconstituted mice i.p. with the hapten 4-hydroxy-3-nitrophenyl acetyl (NP) (38). To assess the immune response to a T cell-independent Ag, mice received 10 μg of NP25-Ficoll in PBS; to assess the T cell-dependent primary immune response, mice received 100 μg of alum-precipitated NP17-chicken globulin (CG) (39); for the secondary response, mice were reimmunized i.p. with 10 μg of soluble NP-CG on day 42 after the first immunization. Sera were collected on days 0, 7, 14, 21, and 28. To detect the presence of NP-specific Abs, ELISA plates were coated with 5 μg/ml NP-BSA and blocked with

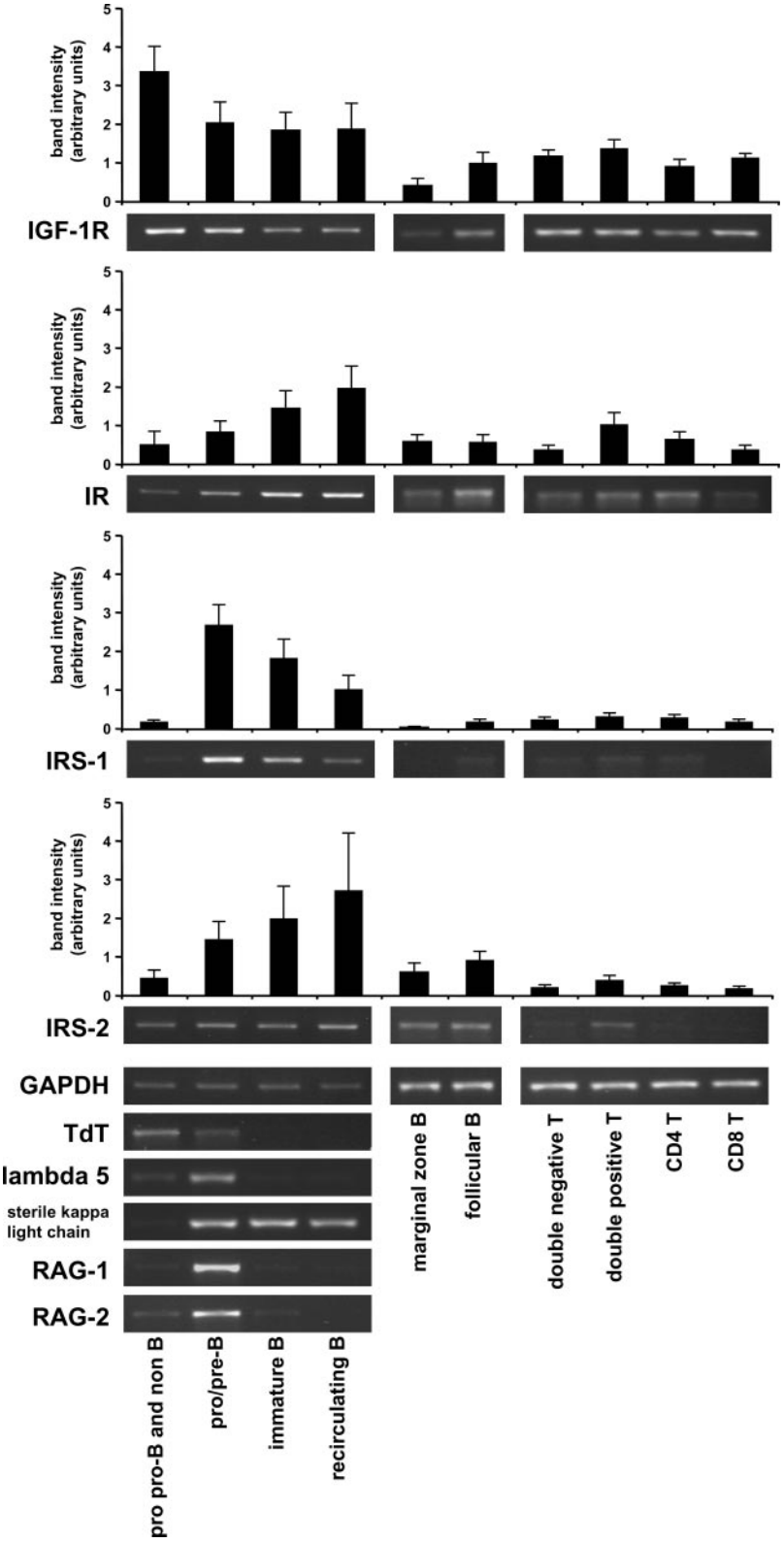

FIGURE 1. Expression of insulin signaling components throughout lymphocyte differentiation. Single cell suspensions of spleen, thymus, and bone marrow of three wt 129/Sv mice were prepared and pooled, and lymphocytes of different developmental stages were sorted by FACS. DNase-treated total RNA was reverse transcribed. PCR for the various signaling components and B cell differentiation markers was performed, and PCR products were resolved on 1% agarose-Tris-acetic acid-EDTA gels stained with ethidium bromide. The figure shows the quantification of three independent experiments normalized on GAPDH expression and a respective gel image.

3% BSA. Sera were added at various dilutions. For the T-independent immune response, IgM, IgG1, IgG3, and γ-bearing Ab levels were determined, and for the T-dependent response, IgG1 and γ-bearing Ab levels were determined using a biotin/alkaline phosphatase-coupled streptavidin system (Roche). Additionally, basal levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were measured.

In vitro Ab secretion

To assess the secretory response of B cells to TI-2 Ag, splenic B cells derived from C57BL/6 mice were plated at 5×10^4 cells/well in 96-well plates and stimulated with dextran-coupled anti-IgD (10 ng/ml) (37), IL-4 (25 U/ml), IL-5 (0.1 ng/ml), and IGF-1 (10^{-7} M) alone or in combination for 7 days in RPMI 1640 medium supplemented with 10% FCS. After collection of the supernatants, Ig levels were determined by ELISA using a biotin/alkaline phosphatase-coupled streptavidin system as described above.

Immunohistochemistry

Cryosections (6 μm) of spleens from reconstituted mice were stained for MZ B cells using rat anti-mouse mucosal addressin cell adhesion molecule-1 (MadCAM-1) Ab (Southern Biotechnology Associates) (40).

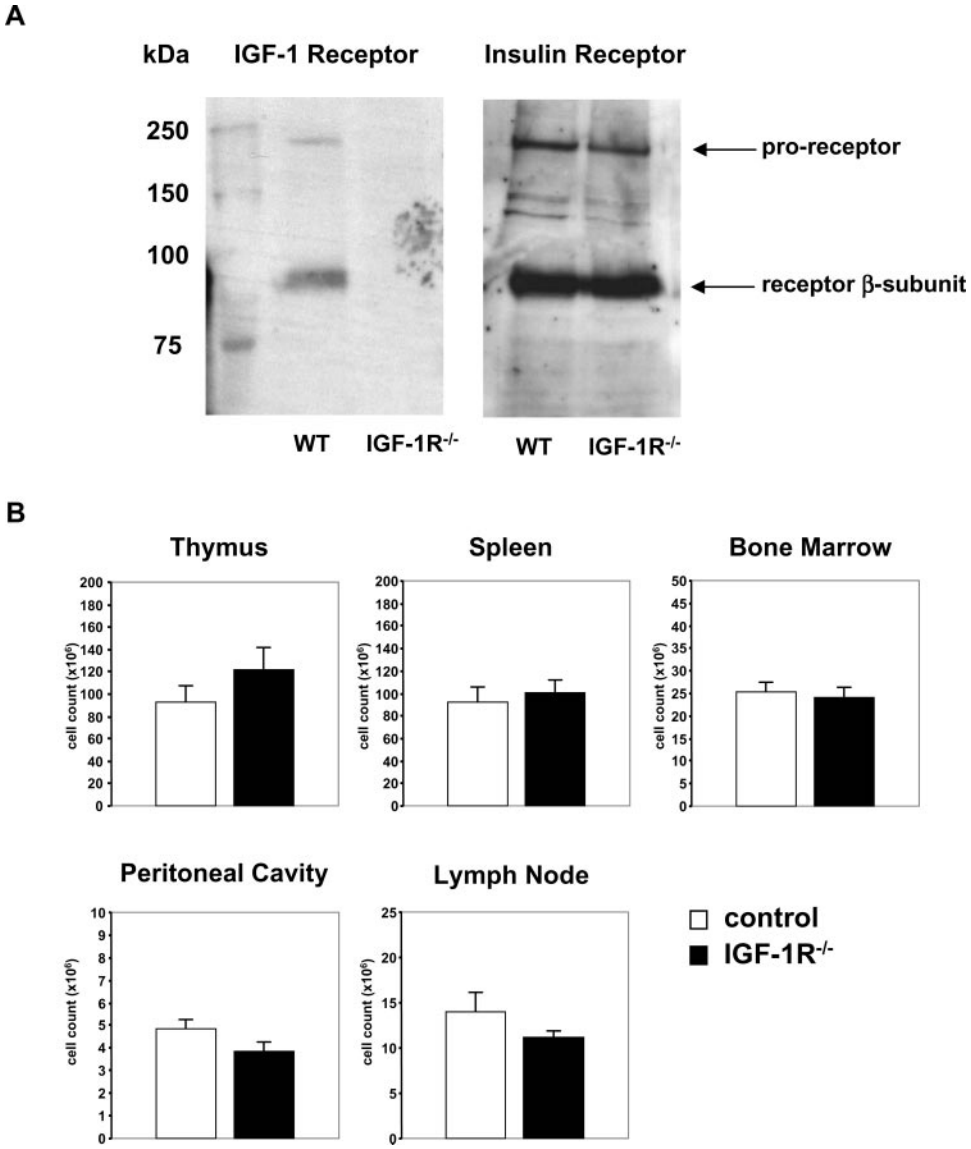

FIGURE 2. *A*, Western blots of protein lysate from control and IGF-$1R^{-/-}$ embryos. Fifty micrograms of whole cell lysates were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The IGF-1R β subunit and IR β subunit were detected with the respective C-20 and C-19 Abs from Santa Cruz Biotechnology and an ECL system. Apparent sizes in kilodaltons of the molecular mass markers (*lane 1*) are indicated on the *left*. *B*, Cell counts in primary and secondary lymphoid organs of reconstituted mice. Total cell numbers in thymus, spleen, bone marrow, peritoneal cavity, and lymph node were determined in all experiments before staining of the cells. Graphs indicate the mean ± SEM (n = 7–10 of each genotype).

Briefly, sections were fixed in acetone, and endogenous peroxidase was blocked with H_2O_2 in PBS and 0.5% BSA. Blocking was performed with 10% FCS in PBS. Bound anti-MadCAM-1 was detected with peroxidase-conjugated goat anti-rat IgG and diaminobenzidine.

Results

Expression of insulin signaling components during murine lymphocyte development

Because previous studies have analyzed the expression of the IGF-1R during lymphocyte development mainly in human cells, we first determined the expression of insulin signaling components during murine lymphoid development. Bone marrow-derived cells of two groups of three wt 129/Sv mice were FACS-sorted into pro- and pre- ($IgM^{-}B220^{int}$), immature ($IgM^{high}B220^{int}$), and recirculating B cells ($IgM^{high}B220^{high}$) and $IgM^{-}B220^{low}$ fraction of pro-pro and non-B cells. Total RNA was isolated and expression of IGF-1R, IR, IRS-1, and IRS-2 mRNA was determined by RT-PCR using gene-specific primers. This analysis revealed that expression of the IGF-1R is highest in the pro-pro and non-B cell fraction, but decreases during B lymphocyte differentiation in bone marrow. In contrast, IR and IRS-2 expression show an increase during B lymphocyte differentiation, whereas IRS-1 mRNA is detectable at the highest degree in the pro/pre-B fraction and steadily declines during further B cell differentiation (Fig. 1).

To verify the purity of the sorted B cell subpopulations, PCRs for TdT, λ5 and a 0.8-kb sterile transcript from the κ L chain locus were performed on the cDNAs from pro- and pre-, immature and mature B cells. This analysis confirmed the successful separation into the respective subpopulations.

The analysis of B cells derived from spleen showed expression of IGF-1R in MZ ($B220^{high}CD21^{high}CD23^{low}$) and follicular ($B220^{high}CD21^{high}CD23^{high}$) B cells as well as expression of IR mRNA. Although IRS-1 expression was hardly detectable in both splenic B cell populations, IRS-2 mRNA expression was evident in follicular and MZ B cells (Fig. 1).

To determine the expression of insulin signaling components during T cell development, total RNA was extracted from FACS-purified thymocytes. Expression analysis revealed expression of the IGF-1R in double-negative ($CD4^{-}CD8^{-}$), double-positive ($CD4^{+}CD8^{+}$), CD4 single-positive ($CD4^{+}CD8^{-}$), and CD8 single-positive ($CD4^{-}CD8^{+}$) cells. IR mRNA expression was lowest in double-negative T cells and peaked at the double-positive stage, and CD4 single-positive T cells showed a greater expression of IR

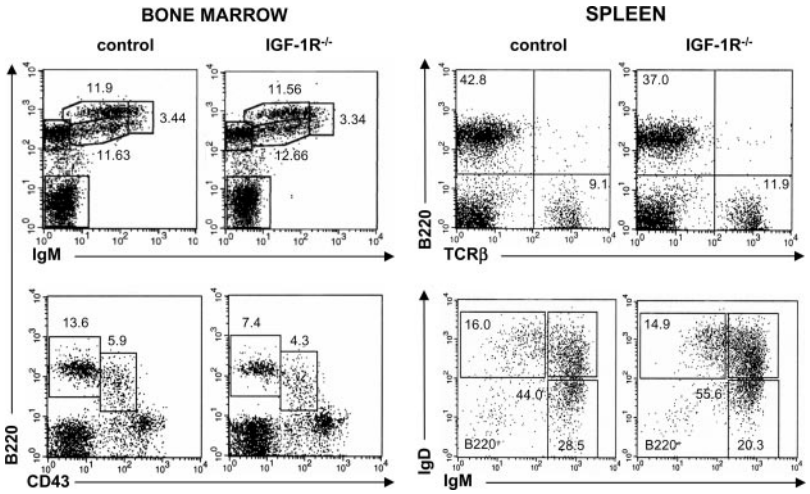

FIGURE 3. Flow cytometric analysis of lymphocytes from bone marrow and spleen of RAG2-deficient mice reconstituted with IGF-1R deficient (IGF-1R$^{-/-}$) or wt FLC. Numbers indicate the percentage of gated cellular subpopulations within the lymphocyte population.

mRNA than CD8 single-positive cells. Compared with this, IRS-1 and IRS-2 mRNAs were only weakly expressed in thymocytes (Fig. 1).

Taken together, this analysis revealed that IGF-1R is the predominantly expressed receptor tyrosine kinase of the insulin family in the main lymphoid compartments, suggesting specific functions of the IGF-1R signaling machinery in lymphocytes.

Generation of chimeric mice lacking functional IGF-1R expression in lymphocytes

To generate mice with IGF-1R$^{-/-}$ T and B cells, IGF-1R$^{-/+}$ mice were mated, and embryos were delivered by cesarean section on day 16.5 postconception. Embryos were genotyped by PCR and 10^6 FLC derived from homozygous IGF-1R$^{-/-}$ or wt littermates were injected i.v. into irradiated recipient animals. Western blot of tissue lysates of the same embryos verified the efficient inactivation of the IGF-1R gene. Expression of the related IR was unaltered (Fig. 2*A*).

Five weeks after FLC transfer, blood was collected from the tail tip, and reconstitution was verified by staining B and T cells. Mice reconstituted with IGF-1R$^{-/-}$ and wt FLC showed similar distribution of blood B and T cells (data not shown), whereas in unreconstituted RAG2-deficient mice, no B and T lymphocytes could be detected (data not shown). Six weeks after transfer, reconstituted mice were killed, and primary and secondary lymphoid organs were isolated. Spleen, thymus, and lymph nodes from IGF-1R$^{-/-}$ and wt reconstituted mice were similar in size and morphology, and cell counts of these organs and of cells derived from bone marrow and peritoneal cavity did not show significant differences (Fig. 2*B*).

Normal B cell development in IGF-1R-deficient chimeras

Previous reports have indicated a role for IGF-1 signaling in lymphocyte development, so cells from lymphoid organs were analyzed by flow cytometry to estimate the influence of IGF-1R deficiency on lymphocyte differentiation. Cells from the bone marrow of IGF-1R$^{-/-}$ and wt reconstituted mice did not show significant differences in the proportions of pro-B cells (IgM$^-$B220^{low}CD43$^+$), pre-B cells (IgM$^-$B220^{int}CD43$^-$), immature B cells (IgM^{high}B220^{int}CD43$^-$), and recirculating B cells (IgM^{high}B220^{high}CD43$^-$; Fig. 3). The amounts of immature IgM^{high}IgD^{low} and mature IgM^{low}IgD^{high} B cells in spleen (Fig. 3) and lymph nodes (not shown) were also similar. Moreover, we detected no difference in the expression of CD19, heat-stable Ag, and the FcγIIR CD32 (not shown). Taken together, we conclude

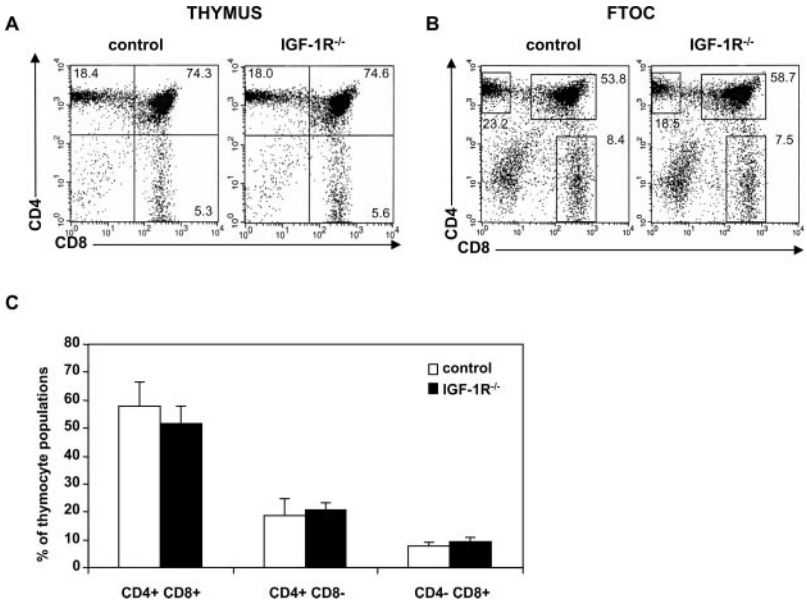

FIGURE 4. Flow cytometric analysis of IGF-1R$^{-/-}$ and control thymocytes. *A*, FACS analysis of thymocytes from reconstituted RAG2-deficient mice. Single cell suspensions were prepared, and thymocytes were stained with fluorochrome-coupled anti-CD4 and anti-CD8 Abs. *B*, FACS analysis of fetal thymi after FTOC. Thymic lobes derived from embryos on ED 16.5 were cultured for 6 days. Numbers indicate the percentage of gated cellular subpopulations within the lymphocyte population. *C*, Proportions of thymocyte subsets after FTOC. Percentages of double-positive (CD4$^+$CD8$^+$) and single-positive (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$) subsets of thymocytes from control (□) and IGF-1R$^{-/-}$ (■) embryos were assessed after 6 days of FTOC. Graphs indicate the mean ± SEM (five controls and four IGF-1R$^{-/-}$).

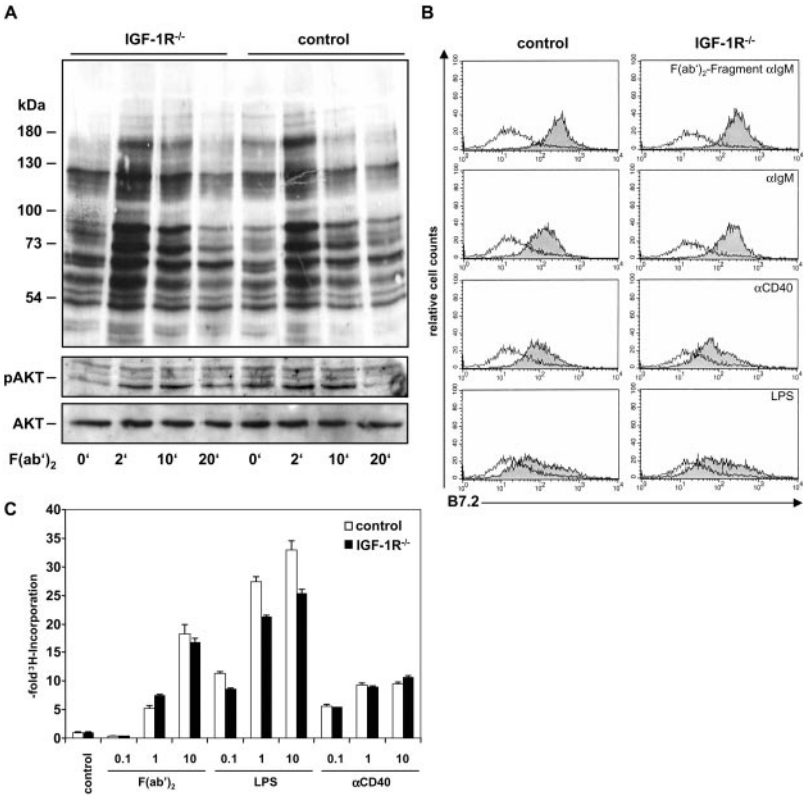

FIGURE 5. In vitro activation of splenic B cells derived from mice reconstituted with IGF-1R$^{-/-}$ or wt FLC. *A*, Anti-IgM induced protein tyrosine phosphorylation in splenic B cells. MACS-purified splenic B cells from mice reconstituted with IGF-1R$^{-/-}$ (*lanes 1–4*) or wt (*lanes 5–8*) FLC were stimulated with goat F(ab′)$_2$ of anti-IgM at 37°C for the indicated time. Whole cell lysates were prepared, resolved on SDS-polyacrylamide gels, and blotted onto nitrocellulose membrane. Tyrosine phosphorylation of intracellular signaling proteins was assessed using the anti-phosphotyrosine Ab PY99 or anti-phospho-Akt. Anti-Akt Ab was used to control for equal loading. The positions of molecular mass markers and their apparent sizes (in kilodaltons) are indicated on the *left*. *B*, Up-regulation of surface CD86 (B7.2) after stimulation with F(ab′)$_2$ of anti-IgM, whole anti-IgM, anti-CD40, or LPS. Representative histograms for three independent experiments show the surface expression levels of CD86 on the surface of MACS-purified splenic B cells from RAG2-deficient mice reconstituted either with wt (control; *left*) or IGF-1R$^{-/-}$ FLC (*right*). Cells were incubated for 24 h with medium in the absence (line) or the presence (filled area) of stimuli. *C*, Proliferative responses of B cells. MACS-purified splenic B cells from RAG2-deficient mice reconstituted with either wt (control; □) or IGF-1R$^{-/-}$ (■) FLC were incubated for 72 h in the presence of the indicated stimuli, and [*methyl*-^{3}H]thymidine incorporation was determined after additional 12–16 h. Proliferation is expressed as fold ^{3}H incorporation compared with unstimulated cells. The graph represents the mean ± SEM of three independent experiments, each performed in triplicate.

that B lymphocyte development occurs independently of IGF-1R expression on B cells.

Unaltered T cell differentiation in IGF-1R-deficient mice

T cell differentiation in wt and IGF-1R$^{-/-}$ reconstituted chimeras was assessed by staining of thymocytes with anti-CD4 and CD8 Abs. Both groups of mice showed similar proportions of double-negative (CD4$^-$CD8$^-$), double-positive (CD4$^+$CD8$^+$), and CD4 (CD4$^+$CD8$^-$) and CD8 (CD4$^-$CD8$^+$) single-positive T cells (Fig. 4*A*). Moreover, numbers of CD4 and CD8 single-positive T cells in spleen and lymph nodes were comparable in these mice, and there were no differences in the expression of CD3ε, TCR αβ, CD44, or the IL-2R α-chain CD25 (not shown). Therefore, T cell autonomous expression of IGF-1R is not required for T cell differentiation.

Previous experiments have indicated a critical role for IGF-1R in T cell differentiation, especially in the transition from the CD4$^-$CD8$^-$ double-negative to the double-positive state, which could be blocked by anti-IGF-1R Abs (15). By contrast, in our analyses of FLC reconstituted mice we could not demonstrate a role for lymphocyte autonomous IGF-1R expression in T cell development. Proper T cell differentiation is thymic epithelium dependent (41, 42), and in the reconstitution model, thymic epithelium is host derived. Hence, the influence of stromal IGF-1R deficiency on T cell differentiation cannot be estimated. To address this issue, we decided to study T cell development in FTOC. We cultured thymi isolated from IGF-1R-deficient embryos and control littermates on ED 16.5 and performed FACS analysis of CD4 and CD8 expression after 6 days in culture. These analyses revealed unaltered T cell differentiation in the absence of functional IGF-1R expression (Fig. 4, *B* and *C*). These data provide clear evidence that nonlymphocyte autonomous IGF-1R expression is not required for thymocyte differentiation.

In vitro B cell activation and protein tyrosine phosphorylation

After stimulation of the BCR, B cells are activated, and an intracellular phosphorylation cascade is initiated. To elucidate the role of IGF-1R expression in the ability of B cells to mount such responses, we purified splenic B cells and induced intracellular tyrosine phosphorylation by cross-linking surface IgM with F(ab′)$_2$ of goat anti-mouse IgM. The pattern of phosphorylated proteins recognized by PY99 Ab was similar in IGF-1R-deficient and wt B cells (Fig. 5*A*). Analysis of intracellular signaling cascades, such as the activation of protein kinase B/Akt (Fig. 5*A*) and JNK (not shown), also showed no difference. These results indicate that B

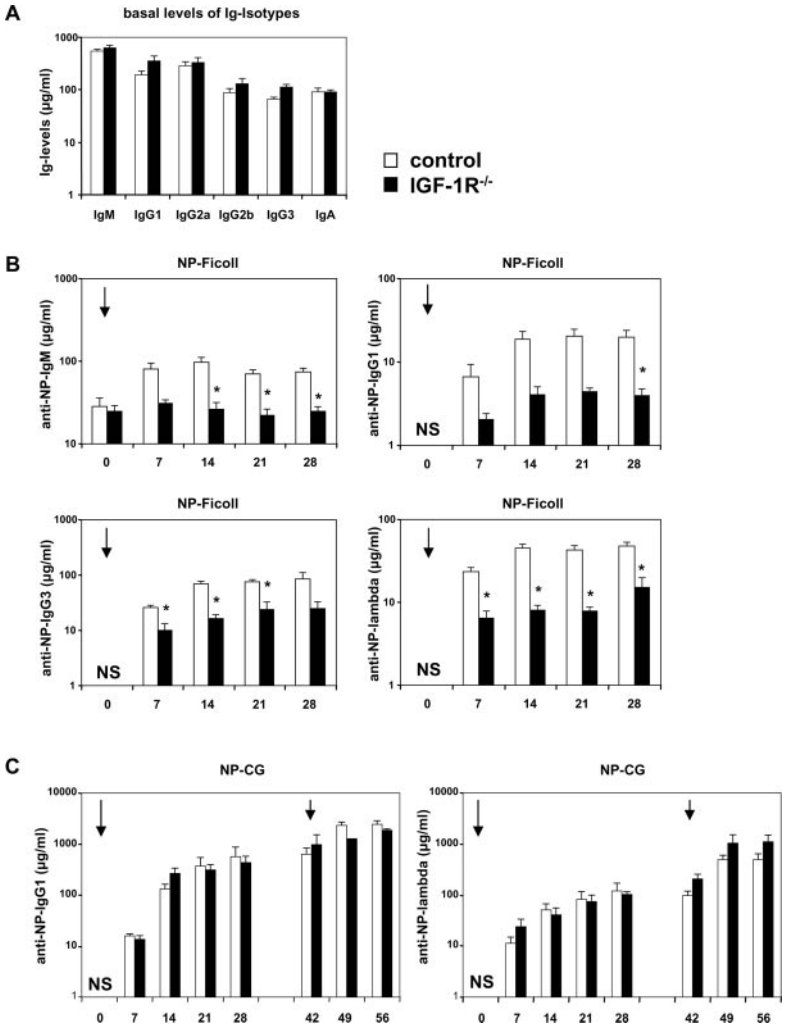

FIGURE 6. Isotypes of serum Ig levels in RAG2-deficient mice reconstituted with wt (control; □) or IGF-1R-deficient (IGF-1R$^{-/-}$; ■) FLC. Bars indicate the mean ± SEM; significant differences ($p < 0.05$) are marked with an asterisk. NS, no signal. *A*, Basal levels of Ig isotypes before immunization. *B*, NP-specific Abs in the sera of reconstituted mice immunized with the T cell-independent Ag NP-Ficoll. The titers of NP-specific IgM, IgG1, IgG3, and γ-bearing Abs were determined in four mice per group on days 7, 14, 21, and 28 after immunization. *C*, NP-specific IgG1 and γ-bearing Abs in the sera of mice immunized with NP-CG as T cell-dependent Ag. For determination of the primary immune response, sera of three or four mice per group were collected 7, 14, 21, and 28 days after immunization. For the secondary immune response, the same mice were reimmunized i.p. on day 42 after the first immunization, and sera were collected on days 7 and 14.

cells from IGF-1R$^{-/-}$ chimeras retain functional signaling capacities after BCR activation, and consequently, BCR-activated signaling occurs independently of IGF-1R expression.

To further address the ability of IGF-1R-deficient B cells to undergo activation, we determined the expression of CD86 in response to B cell activation. Cross-linking of IgM, CD40 (Ab-mediated), or CD14 (with LPS from *E. coli*) led to increased expression of CD86 (B7.2; Fig. 5*B*) and MHC class II (not shown) on the surface of IGF-1R$^{-/-}$ B cells, and this increase was comparable to that seen in cells derived from control mice. Along this line, the proliferative response of splenic B cells to BCR cross-linking and CD40 stimulation over a range of different concentrations was also unaltered in the absence of functional IGF-1R expression (Fig. 5*C*).

Decreased T cell-independent B cell response in IGF-1R$^{-/-}$ chimeric mice

To determine whether chimeric mice develop functional lymphocyte subpopulations and are able to mount normal immune responses, mice were immunized with a T cell-dependent (NP-CG) (39) or T cell-independent (NP-Ficoll) Ag (38). Before immunization, sera derived from both groups of mice showed similar levels of Ig isotypes (Fig. 6*A*). Astonishingly, after immunization with the T cell-independent Ag NP-Ficoll, serum levels of NP-specific IgM, IgG1, IgG3, and λ-chains in IGF-1R$^{-/-}$ chimeric mice were considerably lower than those in the control group (Fig. 6*B*), indicating a disturbed response to the hapten Ag in IGF-1R-deficient B cells. NP-Ficoll belongs to TI-2 Ags, and the data presented above indicate that functional IGF-1R expression is required for establishment of a full immune response to TI-2 Ags. In contrast, the primary immune response to NP in NP-CG-immunized mice was undisturbed in mice reconstituted with IGF-1R-deficient FLC, and secondary immune responses did not show significant differences (Fig. 6*C*).

The response to TI-2 Ags is mainly dependent on B-1 B cells and B cells in the splenic MZ (43–45). To determine whether these lymphocyte populations are present in reconstituted mice in the absence of IGF-1R expression, we FACS-analyzed peritoneal B-1 and splenic MZ B cells derived from these mice. Similar numbers of B-1 (CD19^{high}IgM^{high}CD43^{high}) and CD5^{high} B-1a B cells were detectable in the peritoneal cavity of both groups of mice (Fig. 7*A*). Furthermore, both groups had comparable B cell populations in spleen marginal zones (B220^{high}CD21^{high}CD23^{low}; Fig. 7*B*). Immunohistochemical analysis of splenic cryosections using an anti-mouse MadCAM-1 Ab showed comparable structural organization of MZs in IGF-1R$^{-/-}$ and control chimeras (Fig. 7*C*). Consequently, the reduced TI-2 response in immunized mice is not due to lack of the relevant lymphocytes in IGF-1R$^{-/-}$ reconstituted mice or to a visible disorganization of lymphoid anatomy.

IGF-1 promotes Ig production in an in vitro model of TI-2 response

Because mice reconstituted with IGF-1R$^{-/-}$ FLC showed decreased Ab production in response to TI-2 Ag NP-Ficoll in vivo, although MZ and B-1 B cell populations were normal, we decided

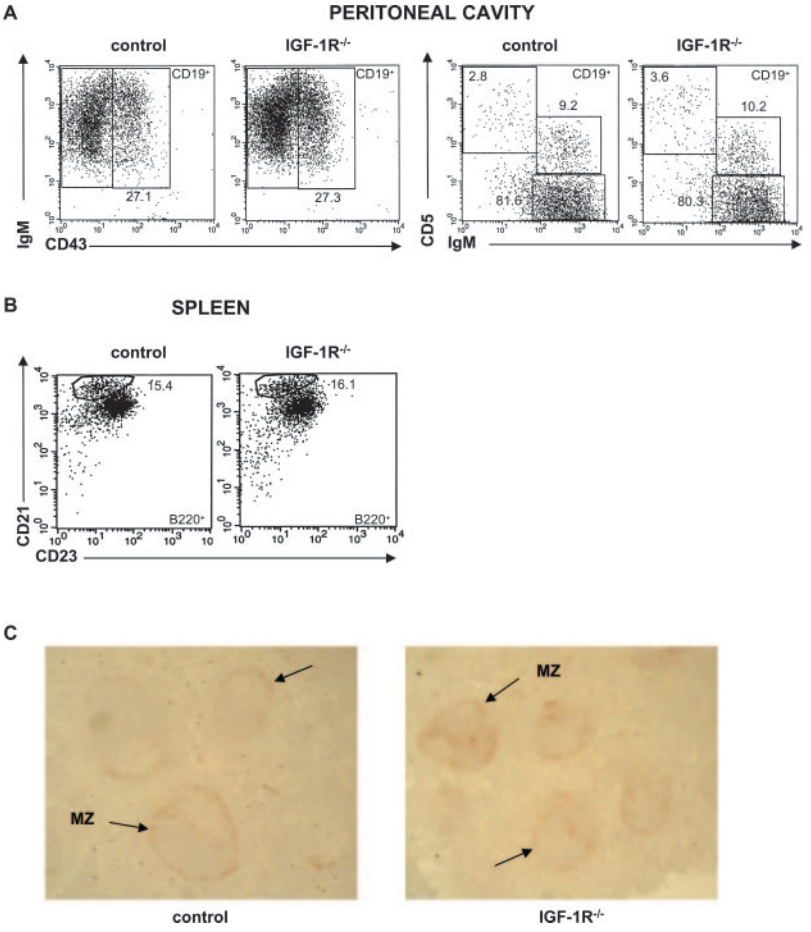

FIGURE 7. B1-B cells and splenic MZ B cells in reconstituted mice. *A*, Peritoneal lymphocytes from mice reconstituted with IGF-1R$^{-/-}$ or control FLC were collected by peritoneal lavage with PBS; stained with anti-IgM, anti-CD5, and anti-CD43 Abs; and analyzed by FACS. *Left*, CD19^{high}IgM^{high}CD43^{high} B-1 B cells. *Right*, CD5^{high} B-1a B cells. *B*, FACS analysis of splenic MZ B cells. Splenocytes were stained with anti-B220, anti-CD21, and anti-CD23 Abs. Graphs show B220^{high}CD21^{high}CD23^{low} MZ B cells in the B220^{+} gate. Numbers indicate the percentage of gated cellular subpopulations within the lymphocyte population. *C*, Immunohistochemical analysis of spleens from chimeric mice. Cryosections (6 μm) were stained for MZs with rat anti-mouse MadCAM-1, peroxidase-conjugated goat anti-rat IgG, and diaminobenzidine. Arrows indicate dark-stained MadCAM-1-positive MZs.

to investigate whether IGF-1 is able to promote Ig production in an in vitro model for TI-2 responses. Previous studies have demonstrated that multivalent cross-linking of membrane IgD and specific costimulation with IL-4 and IL-5 serve as such a model for TI-2 responses (46, 47). Therefore, we incubated purified splenic B cells from wt C57BL/6 mice with a dextran-coupled anti-IgD mAb ($\alpha\delta$-dex), IL4, IL-5, and IGF-1, alone or in combination (Fig. 8). This analysis revealed that in combination with multivalent IgD cross-linking and IL-5, IGF-1 greatly increases IgM secretion in B cells, and this response significantly surmounts the response to $\alpha\delta$-dex with either cytokine alone. To test whether this is due to IGF-1-stimulated proliferation of these cells, we performed B cell proliferation assays under the same conditions. This analysis revealed that IGF-1 did not enhance $\alpha\delta$-dex-stimulated cell cycle progression. We conclude that IGF-1 specifically enhances Ig production, and that this effect is not due to IGF-1-induced B cell proliferation.

Discussion

Signaling through receptors of the IR tyrosine kinase family has been implicated in the regulation of multiple functions in the immune system, particularly in the development and differentiation of T cells. The results of the present study indicate that in vivo, IGF-1R expression on lymphocytes is not required for their differentiation, because in analyses of IGF-1R$^{-/-}$ chimeras, we could not find any defects in the differentiation of T and B cells.

Differentiation of lymphocytes is highly dependent on contact with stromal cells of the respective lymphoid organs (41, 42, 48, 49), and in the reconstitution model, such stromal cells derive from the FLC recipient, hence bearing the genotype of the host. In view of previous studies demonstrating a block of T cell development by incubation of FTOC with a blocking anti-IGF-1R Ab (15), we investigated whether IGF-1R expression on thymic epithelial cells might be required for T cell differentiation. Therefore, we cultured fetal thymi derived from IGF-1R$^{-/-}$ embryos and control littermates. In this analysis we also could not demonstrate disturbed T cell development in the absence of functional IGF-1Rs. These findings underline the importance of genetic approaches to inactivate genes in vivo to address the importance of gene products in biological processes. Taken together, our data clearly rule out an important role for T cell autonomous and nonautonomous IGF-1R expression in T cell differentiation.

Surprisingly, despite normal B lymphocyte differentiation and activation in in vitro experiments, immunization of IGF-1R$^{-/-}$ reconstituted mice resulted in reduced Ag-specific Ig production in response to a T cell-independent Ag, whereas responses to a T cell-dependent Ag were normal. We provide two lines of evidence for a role of IGF-1 signaling in the regulation of TI-2 immune responses: 1) decreased generation of Ig in response to a TI-2 Ag in IGF-1R$^{-/-}$ chimeras in vivo, and 2) the ability of IGF-1 to enhance Ig production after treatment of B cells with a TI-2-like stimulus.

After ligand binding, IGF-1Rs recruit and phosphorylate IRS proteins. This mechanism is not unique for the IGF-1R, but is also common for other hormone and cytokine receptors, namely the IL-4R. Previous studies have provided a specific role for IL-4 in mediating TI-2 responses. Given that on a molecular level IGF-1 and IL-4 signaling pathways converge, it is tempting to speculate that growth factor and cytokine use the same mechanism to regulate the TI-2 immune response. This is supported by our finding

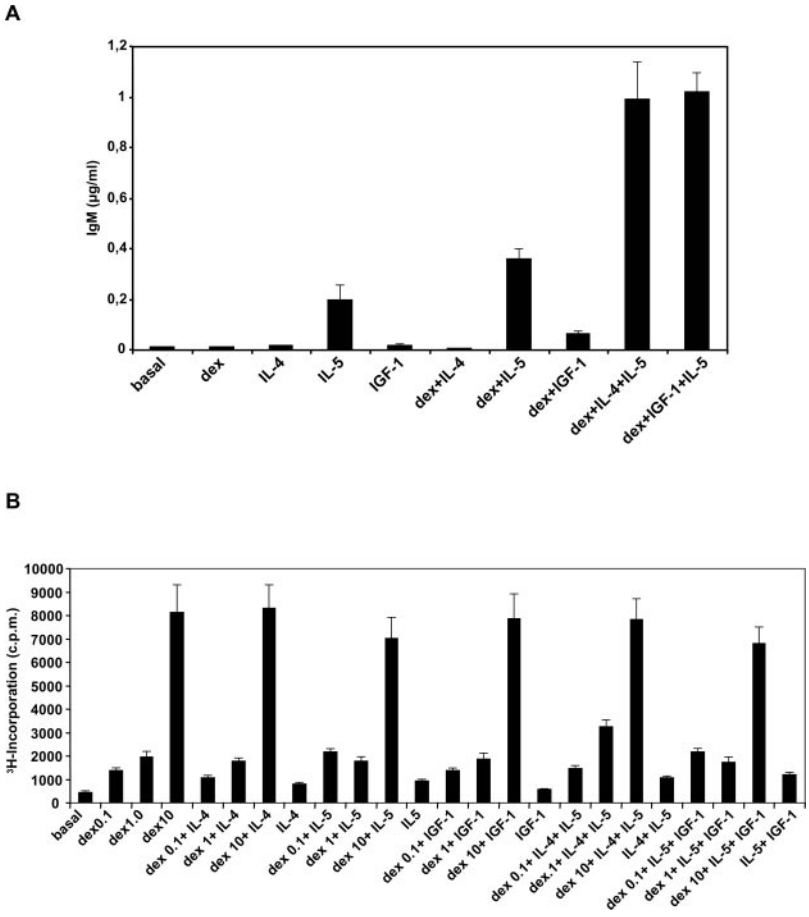

FIGURE 8. In vitro Ig production in a TI-2 model of B cell stimulation. *A*, IgM titers in the supernatants of cultured B cells. Purified splenic B cells from C57BL/6 mice were stimulated with dextran-coupled anti-IgD (10 ng/ml), IL-4 (25 U/ml), IL-5 (0,1 ng/ml), and IGF-1 (10^{-7} M), alone or in combination as indicated. Graphs show a representative of three independent experiments, each performed in triplicate. Bars indicate the mean ± SEM. *B*, [*Methyl*-^{3}H]thymidine incorporation of purified splenic B cells after stimulation with dextran-coupled anti-IgD (0.1, 1, and 10 ng/ml), IL-4 (25 U/ml), IL-5 (0.1 ng/ml), and IGF-1 (10^{-7} M) alone or in combination. Graphs indicate the mean ± SEM of three independent experiments, each performed in triplicate.

that IGF-1 could enhance αδ-dex- and IL-5-stimulated IgM production in purified B cells as potently as IL-4.

Furthermore, IRS proteins are the mediators for both IGF-1- and IL-4-stimulated activation of the PI3K pathway (50, 51). Interestingly, it has been demonstrated that PI3K signaling positively regulates TI-2 responses in vivo; thus, impaired IGF-1R signaling and consequently reduced PI3K activation could lead to a disturbed TI-2 response.

Another substrate of the IGF-1R is the Grb2-associated binder 1 (Gab-1), that is tyrosine phosphorylated upon IGF-1 binding (52). It has been shown that a deficiency in Gab-1 causes an increased Ig secretory response after stimulation with αδ-dex Abs or TI-2 immunization of chimeric mice lacking Gab-1 in B and T cells (53). This study demonstrated that the ability of Gab-1 to recruit Src homology region 2 domain-containing phosphatase 1 is required for its inhibitory role in TI-2 responses. Our previous work showed that Gab-1 overexpression in IRS-1-deficient embryonic fibroblasts strongly enhanced activation of the MAPK pathway (52). Thus, Gab-1 and IRS-2 could act as competitive substrates for the IGF-1R and, as outlined above, potentially the IL-4R. Gab-1 deficiency might thereby enhance the ability of IGF-1 to activate the PI3K pathway and consequently TI-2 responses.

Given the convergence of IGF-1 and IL-4 signaling pathways at the level of IRS proteins, genetically determined insulin resistance, as present in patients suffering from type 2 diabetes mellitus, might also influence the ability of IGF-1 and insulin to regulate TI-2 responses in MZ B cells. Along this line, it is clinically well established that type 2 diabetics are poor responders to pneumococcus vaccination, the clinical correlate of the TI-2 immune response. Therefore, insulin-sensitizing drugs might improve this scenario, beyond their direct effect on the improvement of glucose metabolism. Taken together, the present study assigns IGF-1 a cytokine-like action in the control of the immune response against bacterial polysaccharides. Additional dissection of the intracellular signaling pathway used will potentially define a novel target for improved vaccination strategies in diabetic patients.

Acknowledgments

We thank A. Efstradiatis for providing IGF-1R$^{+/-}$ mice, C. Uthoff-Hachenberg for excellent technical assistance, G. Schmall for secretarial assistance, and C. Göttlinger for cell sorting.

Disclosures

The authors have no financial conflict of interest.

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